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13. ABSTRACT *(Maximum 200 words)*

(1)We will first determine the physical limits to speed and size scaling of established biochemical methods for identification of DNA , proteins, and cells . As necessary, we will optimize methods and materials. The object is to establish scaling of the working biochemistry in order to better design devices (below). For the most part, cost and speed will both scale very favorably with miniaturization, however, the materials and detailed chemical mechanism will require optimizations to obtain the potential advantages of integrated devices. Integrated devices also present new problems to biochemical analysis, for example, stemming from high surface-to-volume ratio. The working mechanism of new devices is certain to remain biochemical in nature, but must be optimized for the application. We propose to gather this necessary information on scaling behavior and to confirm new optimizations using microfabricated flow apparatus in combination with conventional apparatus available at the Whitehead Institute. (2)We will then apply the results above to fabricate and test system modules for high-speed identification and quantitative assay of biochemical materials. The main focus will be on optimizing modules for assay speed, with an emphasis on all contributions to the time budget from sample injection to data collection. Appropriate microfabrication and integrated optics technology will be used. The object is to confirm scaling in analysis time and sample size in highly functional devices. Qualification of the new devices for fast forensic and biochemical assay applications will be made at the DoD application centers (specifically at the Armed Forces Institute of Pathology (APP) The US Army Materials Research Institute for Infectious Diseases (USAMRIID), and at the Aberdeen Proving Grounds (ERDEC).

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Final Report

Program Structure

We propose a two-part program:

(1) We will first determine the physical limits to speed and size scaling of established biochemical methods for identification of DNA , proteins, and cells . As necessary, we will optimize methods and materials. The object is to establish scaling of the working biochemistry in order to better design devices (below).

For the most part, cost and speed will both scale very favorably with miniaturization, however, the materials and detailed chemical mechanism will require optimizations to obtain the potential advantages of integrated devices. Integrated devices also present new problems to biochemical analysis, for example, stemming from high surface-to-volume ratio. The working mechanism of new devices is certain to remain biochemical in nature, but must be optimized for the application. We propose to gather this necessary information on scaling behavior and to confirm new optimizations using microfabricated flow apparatus in combination with conventional apparatus available at the Whitehead Institute.

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Statement of Work

• Establish scaling of the working biochemistry for applications in integrated microfluidic devices. Develop methods, materials and protocols for real-time, DNA-, protein-, and cell-typing.

• Using microfluidic apparatus in conjunction with optical tweezer and molecular imaging apparatus optimize working biochemical separations and detection.

• Develop new sensor materials specifically for integrated fieldable devices, particularly for typing of proteins and cells, (sensing of proteins in order to determine physiological state and biochemical expression, and cells to determine biochemical agents)

• Reduce sensor cost, through reduction of volumes of enzymes, antibodies and reporting materials - now the dominant cost of DNA and immuno assay analysis.

• Develop and test a high-speed capillary thermal cycler module

• Develop DNA amplification and hybridization reactions approaching physical/chemical limits using chemistry and fully functional devices optimized for speed.

Goals relative to current state of the art: Improve the speed of hybridization sensors by one to two orders of magnitude, PCR amplifiers by one order of magnitude. Demonstrate 60X improvement in the rate of DNA typing via electrophoresis. Improve detection efficiency for small protein samples by approximately two orders of magnitude.

Detailed Technical Approach and Work Plan
Materials and Device Scaling

We will first establish scaling of the working biochemistry for applications in integrated microfluidic devices and develop methods, materials and protocols for real-time, DNA- , protein- , and cell-typing. We will then apply this information in series of miniaturized devices.

Scaling of current analysis methods

This task will emphasize scaling of the methods and materials of current assay. We will initially focus on methods of DNA typing and sequencing, since these procedures have found extensive application in biomedicine and forensics. The method will be to construct a heavily instrumented microflow-cell apparatus and to gather data efficiently as reagents and treatments are varied from those used in current macroanalysis. We will construct this microflow apparatus as a series of microdevices that can be inserted into our existing molecular imaging/laser tweezer. The optical tweezer has sufficient optical resolution and time response to dynamically image single fluorescently tagged DNA molecules in the flow stream of a microfabricated device.

Reduce sensor cost through (many order of magnitude) concentration and volume scaling

As mentioned above, at most centers reagents currently dominant the cost in DNA sequencing. Reasonable extrapolation of trends intensifies concern that reagents will increasingly dominate costs and will be a severe constraint in the *applications* of DNA typing and sequencing. The smallest reagent amount which can be used for a single sequencing reaction is, in theory, given by the amount needed for loading and detection by the gel electrophoresis system. In the case of the routinely used ABI sequencing gel with 200 μm thickness and 34 cm length 2 μL of sample have to be applied per lane. The actual volume used will be larger however because of the specific needs of the up front sample handling. The size of the smallest sample handling volume is limited by a) the smallest volume which can be pipetted accurately (still restricted as practiced by human pipetters) b) uncontrolled changes in concentration due to rapid evaporation of small volumes or sample losses due to non-adsorption to vials, pipette tips, etc., and c) specific needs of the reaction itself (e.g. an essential purification step might only be applicable with a certain volume). By using microfabricated structures and robotics we will decrease the loading volume for each sequencing reaction to a 10 nL. This is an improvement of a factor of 200 compared to the ABI slab gel loading volume. This can in theory bring down the reagent cost by the same factor. However, to take full advantage of this improvement, the sample-handling volume has to be decreased in parallel which means the entire upfront sample treatment procedure has to be miniaturized and adjusted to the needs of the micro separation system. This, in turn, implies that pipetting systems capable of handling accurately sample volumes in the nL range have to be developed. Our approach will be to use piezo elements (used in ink jet printers), and electrophoretic transfer (by drawing ion currents to dispensing tips) for the controlled dispense of the single reagents into the reaction vessel and for the controlled loading of the finished reaction onto the gel. Second, to accommodate this small sample volumes, entirely new microreactors (micro titer plates) may have to be developed. Such plates might be either micromachined into glass or fused silica or molded into plastic. In parallel, all the upstream DNA reaction/ purification steps will be adjusted to smaller volumes or replaced by equivalent small volume procedures.

Silicon device processing will take place microfabrication facilities located at the Whitehead Institute and MIT. 4" silicon and quartz substrates will be used as starting materials. Silicon substrates will be oxidized to 2 μm thickness in a Bruce BDF-4 furnace system. This will be followed by Ti/AlSi/Ti deposition in a Varian 3180 sputter deposition system. First level metallization will be patterned using a Solitec 5110 photoresist spin coater and a Karl Suss MA 4 contact aligner. These wafers will have electrode etches performed on a Lam Research 680 metal dry etcher followed by resist ashing in a Drytek Megastrip 6 photoresist stripper. This metallization will be sintered, followed by a 1.5 μm PECVD silicon dioxide deposition in a Plasma Technologies Plasmalab. Vias will be patterned using the Karl Suss MA4, followed by plasma oxide etching in a Lam Research 594 and resist ash. Second-level Ti/AlSi/Ti bridge metal will be sputtered on the Varian 3180, followed by contact patterning and plasma etching in the Lam Research 680. After resist strip and metal sinter, 2.2 μm of PECVD oxide will be deposited in the Plasma Technologies Plasmalab. After defining electrochemical electrode contacts through contact aligning, a deep oxide etch through both layers of PECVD oxide down to the underlying first metal will be performed. The electrode regions will be re-patterned for lift-off and a Ti/Pt layer will be sputtered as the electrochemical contact. Following Ti/Pt lift-off and wafer polishing, packaging will be performed using glass etching in a second wafer to define channels followed by ultrasonic direct bonding to the first wafer. Layout for these devices will continue to be performed in house as described earlier. Additional masks will be purchased from Photronics Sunnyvale (formerly Hoya MicroMask).

Large area microfabricated devices (6 inch to 12 inch dimensions) will be constructed in glass using semiconductor technology and large-area patterning instrumentation supporting the flat panel display industry. Mask design will be performed in KIC, a public domain CAD tool, run on a DEC 5000/200. Initial designs will consist of 384 parallel microchannels with a small region near the end with sidewalls. Sharp tapers of the sidewalls will be included to allow defect-free gel slide-casting. This design will be converted into CIF and GERBER formats for use in mask fabrication. For glass fabrication, 17" square plates will be obtained from Image Quest as 1000A nichrome on glass. These plates will be exposed to SSI flat-panel photolithography equipment, which operate on step/repeat patterning and field stitching. Plates will be shipped to the Whitehead Institute for timed isotropic etching (nominally 00:13:20 at 3 μm/min) in nitric acid:HF solution in a large volume bath under agitation. After rinse, this etch will be followed by a nichrome etch in ceric ammonium nitrate. This substrate will be transferred to a large volume rinse bath immediately afterwards to avoid residue formation. Following sample etch, these plates will be diced into 75 mm x 170 mm format with a conventional glass saw, yielding 10 384-lane separation elements per panel. If the projected single-base electrophoretic resolution is not achievable in the 170 mm format, a mask revision will permit changing the separation length to devices as long as 400 mm.

High -Speed Capillary Thermal Cycler

It is well established that the thermal cycling apparatus, not the intrinsic enzyme activity rate, determines the speed of many technologically important thermal cycle reactions, including the polymerase chain reaction (PCR). We will develop a device module which will reduce the thermal equilibration time of the sample by several orders of magnitude relative to the current best cyclers. As pointed out by the previous researchers, reduction of the ramp times in thermal cycling should both reduce total reaction times (proportionately to the time saved) and also in a non-linear manner due to potential improvement in the PCR yield (which exponentiates with each cycle). Our

objective is to totally eliminate instrument-related limits on PCR time, and to thereby make direct observations and optimizations on the intrinsic chemistry.

The concept of the capillary thermal cycler is illustrated in Fig.3. Static capillary thermal cyclers and microfabricated-well thermal cyclers have been developed in the past. The unique features of our design are that there will be zero apparatus-related thermal mass (in temperature cycling), there will be very high surface contact area, and we will use shear-fold mixing by dynamically oscillating the sample along the capillary wall. Movement between temperature zones is by active servo-controlled flow (in the initial device - pressure driven). The combination will reduce the thermal equilibration time of the sample by several orders of magnitude relative to the current best cyclers. As pointed out by the previous researchers, reduction of the ramp times in thermal cycling should both reduce total reaction times (proportionately to the time saved) and also in a non-linear manner due to potential improvement in the PCR yield (which exponentiates with each cycle). Our objective is to totally eliminate instrument-related limits on PCR time, and to thereby make direct observations and optimizations on the intrinsic chemistry.

The reactions will be carried out inside of a micro capillary with different temperature zones through which the fluid plug will move under the guidance of a high speed close-loop servo (driven by pressure). We will automate the capillary loading and unloading with aspiration via vacuum and/or capillary action, and ejection via pneumatic or piezoelectric action. In addition, we will constantly oscillate the fluid while it is inside of the capillary, providing a shear folding action, known to be an extraordinarily efficient means of stirring (and not possible in the macro domain). The result will be an apparatus with zero apparatus-related thermal mass, with optimized contact surface, and with a microfluidic stirring. The thermalization time will be reduced to the 10-100 microsecond range for full temperature stability. This is an improvement of many orders of magnitude relative to all previous methods (including resistively heated microwells), and will allow probing for the ultimate chemical limits of the amplification chemistry with out limitation from the apparatus.

High-speed microfluidic separation module

In this task we will develop a series of very high speed microfabricated capillary electrophoresis devices. The primary objectives are devices optimized for speed rather than highly parallel devices for volume production of DNA sequence. However, the later devices are of great interest to large genetics centers and will be considered as a possible spin-off in the development.

In previous experiments we have demonstrated useful electrophoretic separation of DNA with a chip in an assay time of 15 minutes. The goal of this element will be to reduce this time for DNA typing applications by a factor of 60 to a run time of 15 seconds or less. This module will go through progressive refinement to permit target identification in the fastest time possible with minimal operator skill. The approach will be to use of short separation distances coupled to small injection volumes allows for a ten fold reduction in separation time with similar resolution to a capillary based instrument. Further gains can be attained by o resolution (and therefore accuracy of identification) for increased speed by moving the detector even closer to the injector. Another factor of six increase in speed should be attainable in this manner (with a loss in resolution of a factor of six). Therefore, separations that currently require 15 to 60 minutes should be reduced to 15 to 60 seconds. In advanced versions of the module sample preparation

will be integrated on the chip, where liquid transfers and mixing are rapid, sample loss is minimal, and operator skill level is unimportant.

In the first generation only the electrophoresis module will be incorporated into the chip. Most of the sample prep (cell lysing, DNA amplification, etc.) will be done in a separate module and transferred by robot to the injection port of the electrophoresis module. Once loaded electrokinetic and/or hydrodynamic forces will be utilized to move reagents and sample through the device. The sample may first be reduced in volume by use of a microfabricated sample splitter or injector. The injection plug will then be combined with enzyme at a "T" intersection and the voltage will be turned off to allow for digestion. After an appropriate period, voltage will again be applied to cause a separation of the DNA fragments as they travel down this channel towards the detector, which would most likely be an LED or laser diode based fluorescence system. In future generations, more of the sample handling will be integrated onto the chip upstream from the separation module in a sample prep module. This sample prep module will consist of many parallel single-use systems to avoid contamination, particularly if PCR is implemented.

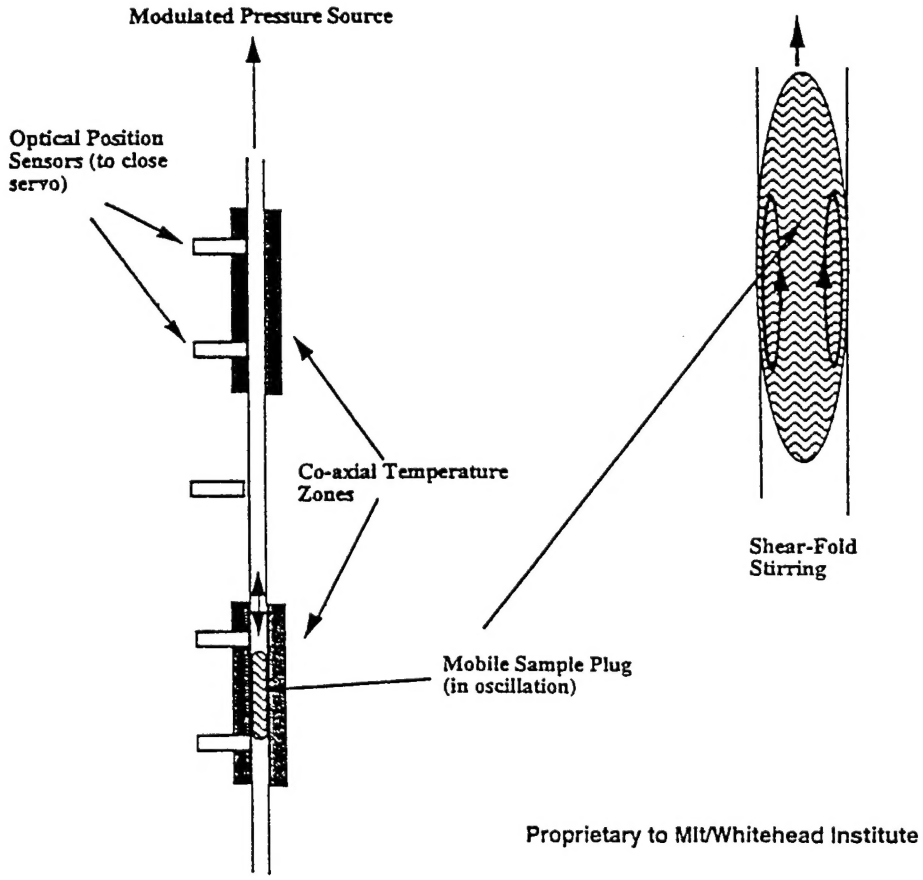

Figure 3: High-speed servo-actuated thermocycler. The design features a capillary with preheated zones. The drop is transferred between zones under close-loop servo driven by a pressure source. The active drop is oscillated rapidly in each heat zone to induce shear-flow mixing. This design removes all instrument contributions to thermalization and will permit optimization of concentrations and reagents for thermal-cycled amplification reactions.

High-Speed Integrated Microfluidics Module

The goal of this element will be to produce a module for DNA typing applications with an electrophoretic run time of 15 seconds or less. This module will go through progressive refinement to permit target identification in the fastest time possible with minimal operator skill. The module will be interfaced with up-stream amplification and restriction enzyme digestion sample preparation. Figure 4 illustrates this device, which we consider to be relatively low risk to build given our previous experience with this class of devices. Obtaining useful results in the context of real analysis is another story. Scaled chemistry, automated sample loading and automated injection of sieving material, high speed sample dispersion and high-speed detection will be combined to achieve time efficient DNA typing in a moderate-resolution electrophoresis device. The elapsed time for this sequence using the best available current technology is many hours. Our objective will be to take samples from 384-well microtiter plates (prepared on the WI/CGR Sequatron robot) to finished assay, including all overhead steps (e.g., gel loading) with a one- to two-order-of-magnitude improvement in elapsed time.

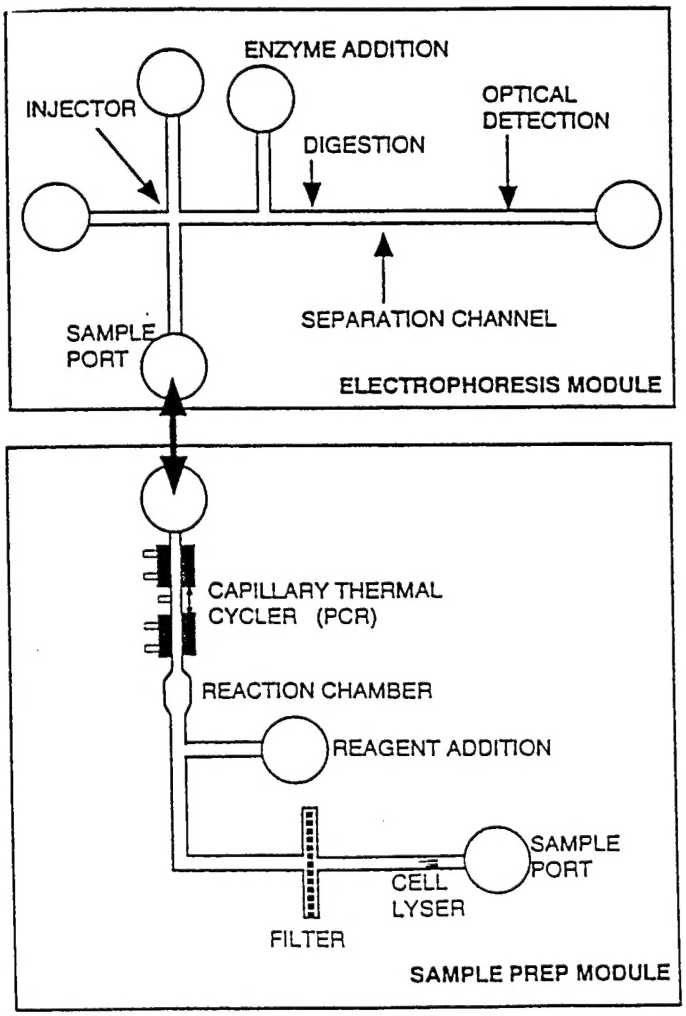

Figure 4: Two-module microfluidic system based on previous work of the researchers. This design will be incorporated as one of the analysis stations and will be extended to high-speed analysis of cells. This device is based on extensive prior work and is low-risk, however, integration into the automated load will permit optimization of chemistry, not heretofore possible and will permit extensive real applications in a clinical setting. The module will be optimized for speed.

Comparison with Other Ongoing Research

High-density MEMS sample injection

Many attempts have been made to achieve low-volume injection into microfabricated channels. Harrison (1992) demonstrated that the intersection volume of crossed microchannels etched in glass could be used successfully to define a plug for protein separation injection. This plug could then be separated by electrophoresis along one of the channels. Later, the so-called "double-T" configuration (Effenhauser, 1993; Seiler, 1994) was used to obtain arbitrary plug length within the separation channel. Post-injection leakage in both of these cases was found to be a significant effect (Fan, 1994) and to date there are still some fluidic effects observed which do not fit the first-order models. It is worthwhile to point out that despite the years of work based on the microchannel techniques first proposed by Harrison et. al., the basic packaging issues for making this a viable technology have still not been solved. Sample loading to the injection arm of the cross is still usually performed by filling a macro-reservoir (such as a pipet tip or a cylindrical portion of microcentrifuge tube) which has been glued to the entrance port. Hence large volumes of sample still need to be presented to the chip; the microfabricated part only serves to move the location of this presentation slightly upstream. In addition, no real reduction in area is achieved with these techniques. Thus, massive parallelism is difficult to achieve. In order to develop a fully functional MEMS-based sequencing system in the next few years, this issue must be given high priority.

PCR Chips

Efforts are underway to develop MEMS style PCR chips. Among these are two very active efforts at Lawrence Livermore Laboratory and at Perkin Elmer Applied Biosystems. Both of these are making notable improvements in the miniaturization and integration of PCR. In the program above we propose a significant variation of PCR which has zero device-related thermal equilibration (theoretic thermal switching time much less than 1 millisecond), with active servo control of the fluidics. Our device should therefore unequivocally reveal the chemistry limited performance of PCR. The PCR device is an important module to have available for integration with our hybridization and electrophoresis modules. We will collaborate with on-going DARPA projects to incorporate the best features of our own designs with the current state of the art.

Discussion of Previous Accomplishments

The MIT/Whitehead Institute group has been actively developing a series of microfabricated devices for electrophoretic separation of DNA. Several of these are shown in Figs. 4 and 5, and are described in previous publications and reports. The silicon devices pictured in Fig. 8 are microfabricated using a five-mask process and feature a novel drive and channel configuration. The device in Fig. 5 is fabricated in simpler two mask process incorporates 256 parallel channels. A third device illustrated in Fig. 6 is a bonded-glass single mask structure. All of these have been tested using laser fluorescence detection. A novel piezo injection method was developed which permitted scaling of the injection volume to 50 pL, a factor of 10,000X reduction relative to the conventional capillary electrophoresis requirement. The device in Fig. 6 was developed for forensic applications and was demonstrated with AFIP as a means to separate short tandem repeat alleles form the HUMTHO1 locus of the human genome following PCR. A 5X improvement in speed (and 10,000X reduction in injection volume) relative to conventional capillary electrophoresis was demonstrated.

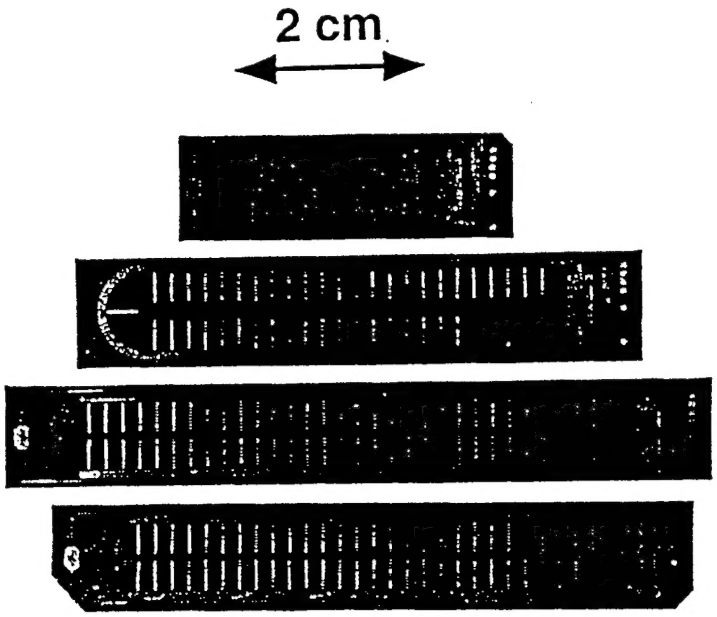

Figure 5: Silicon microelectrophoresis chips. Several designs are represented. All are microfabricated using five-mask lithography to define a complicated reticulated electrode, four-phase drive, with electrode contacts engineered for control of evolved gas, and various channel geometries. Electrodes are Ti/Pt, intermetal dielectric is PCVD glass.

As outlined in the bibliography the team has also had more than three years experience developing hybridization arrays. The novelty of these devices, in the context of other national efforts, (e.g., Affimetrix) is the ability of our chips to do onboard detection through sensing of permativity changes due to hybridization on the array. These devices are more thoroughly described in the references.

For the piezo-electric injection an ink drop dispenser system (Laserguided MicroJet, MicroFab Technologies) was set-up and tested in our laboratory. This nozzle injector is capable of forming and delivering 50 to 100-μm diameter drops to a specified target area. Alignment was achieved by using a red wavelength laser diode to aim the drop stream. The droplet generator which is the heart of the piezoelectrically-driven injector consists of a glass tube surrounded by an annular piezoceramic element. The exit orifice is pinched down to the form of a nozzle (~60 μm diameter) to properly define the equilibrium position of the meniscus. Electrodes are plated on the piezoelectric ceramic. A voltage pulse applied to the electrodes generates a compressive contraction of the glass element and squeezes the incompressible fluid longitudinally in both directions. By providing proper acoustic tuning to the pulse delivered to the device, the drop can be forced through the forward output orifice. In this way, drop-on-demand conditions can be achieved in response to an applied voltage pulse. This same approach is to be developed as one of the robotically controlled dispensing modes described in the program above.

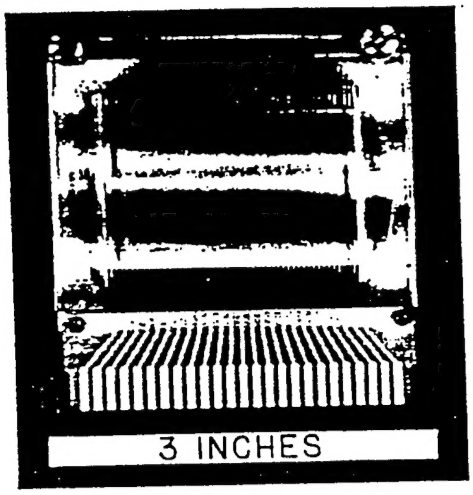

Figure 6: Multichannel glass microelectrophoresis chip. Fabricated using photosensitive polyimide to define straight channels, then a cover plate is thermocompression bonded to seal the device. The object is low-cost fabrication, via a minimum number of processing steps and the use only of automatable processes. Two-mask process.

The Genome Center has had much experience with automation projects, including the Waffle Iron PCR machine (in use for five years and adopted by other Genome Centers), the ROSYS robotic system (throughput of 20,000 samples per day for flexible laboratory operations), the SPLATT (hand-held 96-head parallel pipettor), and the Genomatron system, a custom factory-style automation system for setting up 150,000 PCR reactions in 90 minutes (in routine use for eight months). An automated sequencing

sample handling robot, the "Sequatron", which was completed in July 1995 and is currently being upgraded to a higher grade platform.

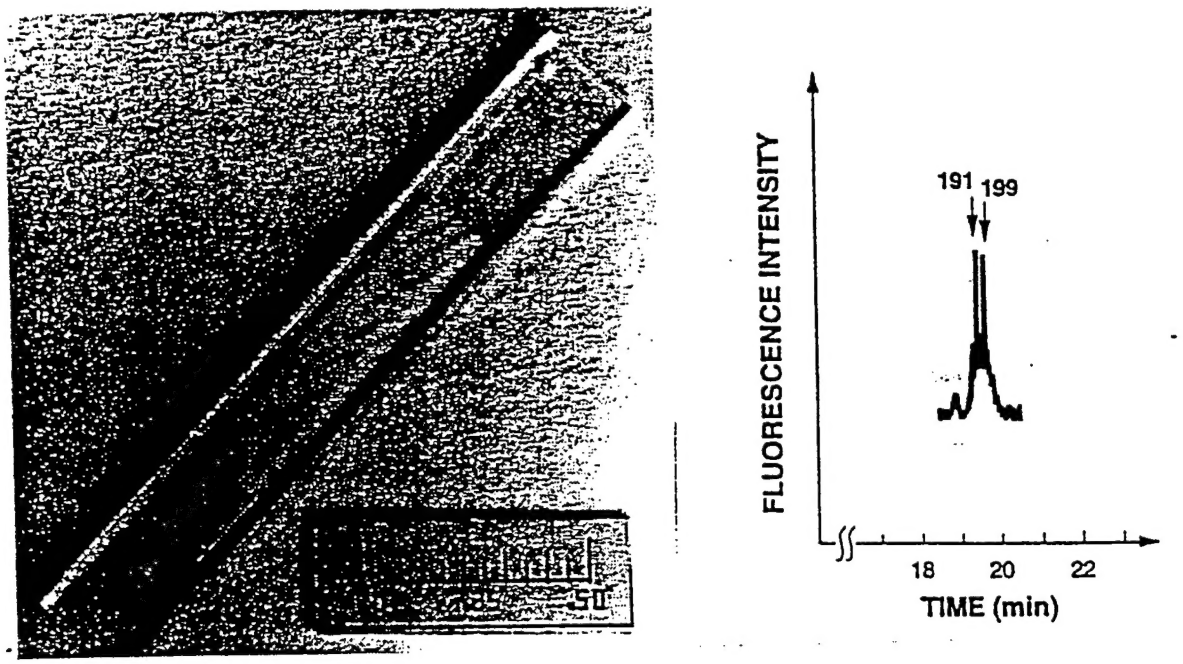

Figure 7 Serpentine, glass bonded microelectrophoresis chip. Fabricated with photoresist, glass etching and glass fusion bonding. This device was applied to separation of short tandem repeat (STR) alleles from the HUMTHO1 using PCR.

Facilities

Matsudaira/ Ehrlich Laboratories

The Matsudaira and Ehrlich laboratories on the 6th and 3rd floors of the Whitehead Institute consist of 6 lab modules (3000 sq. feet) containing 6 lab benches/module, a full size walk in cold room, a small walk in warm room, a specially designed clean room to assemble microfabricated components, and a special instrumentation room (550 sq. feet) for peptide and PNA synthesis, protein purification, and protein analysis. A microscopy suite (2000 sq. feet) on the 4th floor contains microtomes, benches, a hood, and two rooms for a Phillips 410 EM, BioRad MRC600 Confocal microscope, a Scanalytics CELLscan deconvolution-based 3D imaging microscope, a dedicated research video microscope equipped with a 7W IR optical tweezer, and a separate dedicated research CCD camera mounted microscope. All microscopes are equipped with high NA, high and low power objectives for imaging in fluorescence and interference modes. The labs and offices contain Macintosh and IBM clone personal computers that are linked to the Institute VAX minicomputer. The lab is equipped with a Perceptive Biosystems Voyager Elite MS/MS (time of flight mass spectrometer), an HPLC (HP1090M), 2 automated gas phase sequenator (ABI 470/120/900 and Portion 2090E) equipped with on-line amino acid analysis, low pressure columns, column monitors, and fraction collectors, centrifuges (2 RC5B, 1 RC3B, 1RC90, 1 RT-9000 Dupont), power supplies, gel apparatus (protein & DNA sequencing), refrigerators, freezers (-20° and - 70°), chemical hoods, and a video microscope. There are shared instrumentation rooms on the floor

containing common use gel dryers, lyophilizers, speedvacs, balances, spectrophotometer, Elisa plate reader, centrifuges, fluorimeter, Milli Q water system, a shared chemical storage room, darkrooms.

Whitehead Institute Center for Genome Research There is 16,500 square feet of laboratory space dedicated to the MIT/Whitehead Genome Center. This space includes bench space, shared rooms for radioisotope handling, consistent temperature (4°C and 37°C) rooms, darkrooms, and heavy equipment rooms.

The CGR acts as one of the central DNA mapping and sequencing facilities for the NIH as part of the Human Genome Project and also in multiple genetics research and technology projects for numerous sponsors. It is equipped with extensive commercial robotic fluid handling automation and currently operated six automated Perkin/Elmer Gel Plate sequencers at near full capacity. The CGR is also a major beta cite for evolving DNA assay technology with numerous commercial prototype sequencers and robots.

The CGR has a network of 18 UNIX workstations and several Macintosh personal computers connected through Ethernet. Outside connections provide the Center with full access to computer services at the Whitehead's computing facilities (e.g. Medline; VAX mainframe). These connections also provide access to information sources at MIT and the world via the Internet. A PC set-up for automatic visual entry of dot blot data with CCD camera and a Montage film recorder are also available.

There are low and medium speed centrifuges, preparative ultracentrifuges, scintillation counters, incubators for bacteria, liquid nitrogen freezers, laminar flow hoods, microscopes, electrophoresis and sequencing facilities and PCR machines available.